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Research paper

Sequence-activity relationship, and mechanism of action of mastoparan analogues against extended-drug resistant *Acinetobacter baumannii*

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ABSTRACT

The treatment of some infectious diseases can currently be very challenging since the spread of multi-, extended- or pan-resistant bacteria has considerably increased over time. On the other hand, the number of new antibiotics approved by the FDA has decreased drastically over the last 30 years. The main objective of this study was to investigate the activity of wasp peptides, specifically mastoparan and some of its derivatives against extended-resistant *Acinetobacter baumannii*. We optimized the stability of mastoparan in human serum since the specie obtained after the action of the enzymes present in human serum is not active. Thus, 10 derivatives of mastoparan were synthetized. Mastoparan analogues (guanidilated at the *N*-terminal, enantiomeric version and mastoparan with an extra positive charge at the *C*-terminal) showed the same activity against *Acinetobacter baumannii* as the original peptide (2.7 μM) and maintained their stability to more than 24 h in the presence of human serum compared to the original compound. The mechanism of action of all the peptides was carried out using a leakage assay. It was shown that mastoparan and the abovementioned analogues were those that released more carboxyfluorescein. In addition, the effect of mastoparan and its enantiomer against *A. baumannii* was studied using transmission electron microscopy (TEM). These results suggested that several analogues of mastoparan could be good candidates in the battle against highly resistant *A. baumannii* infections since they showed good activity and high stability.

1. Introduction

Infections caused by multidrug resistant bacteria are currently an important problem worldwide. Taking into account data recently published by the WHO, lower respiratory infections are the third cause of death in the world with around 3.2 million deaths per year, this number being higher compared to that related to AIDS or diabetes mellitus [1]. It is therefore important to solve this issue, although the perspectives for the future are not very optimistic. During the last 30 years an enormous increase has been observed of superbugs isolated in the clinical setting, especially from the group called ESKAPE (***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, ***E****nterobacter* spp.) which show high resistance to all the antibacterial agents available [2]. We will focus on *Acinetobacter baumannii*, the pathogen colloquially called “iraquibacter” for its emergence in the Iraq war. It is a Gram-negative cocobacillus and normally affects people with a compromised immune system, such as patients in the intensive care unit (ICU) [3,4]. Together with *Escherichia coli* and *P. aeruginosa*, *A. baumannii* are the most common cause of nosocomial infections among Gram-negative bacilli.

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The options to treat infections caused by this pathogen are diminishing since pan-drug resistant strains (strains resistant to all the antibacterial agents) have been isolated in several hospitals [5]. The last option to treat these infections is colistin, which has been used in spite of its nephrotoxic effects [6]. The evolution of the resistance of *A. baumannii* clinical isolates has been established by comparing studies performed over different years, with the percentage of resistance to imipenem being 3% in 1993 increasing up to 70% in 2007. The same effect was observed with quinolones, with an increase from 30 to 97% over the same period of time [7]. In Spain the same evolution has been observed with carbapenems; in 2001 the percentage of resistance was around 45%, rising to more than 80% 10 years later [8]. Taking this scenario into account, there is an urgent need for new options to fight against this pathogen. One possible option is the use of antimicrobial peptides (AMPs) [9–11], and especially peptides isolated from a natural source [12]. One of the main drawbacks of using peptides as antimicrobial agents is the low stability or half-life in human serum due to the action of peptidases and proteases present in the human body [13], however there are several ways to increase their stability, such as using fluorinated peptides [14,15]. One way to circumvent this effect is to study the susceptible points of the peptide and try to enhance the stability by protecting the most protease labile amide bonds, while at the same time maintaining the activity of the original compound. Another point regarding the use of antimicrobial peptides is the mechanism of action. There are several mechanisms of action for the antimicrobial peptides, although the global positive charge of most of the peptides leads to a mechanism of action involving the membrane of the bacteria [16]. AMPs has the ability to defeat bacteria creating pores into the membrane [17], also acting as detergents [18], or by the carpet mechanism [19]. We have previously reported the activity of different peptides against colistin-susceptible and colistin-resistant *A. baumannii* clinical isolates, showing that mastoparan, a wasp generated peptide (H-INLKALAALAKKIL-NH_2), has good *in vitro* activity against both colistin-susceptible and colistin-resistant *A. baumannii* [20]. Therefore, the aim of this manuscript was to study the stability of mastoparan and some of its analogues as well as elucidate the mechanism of action of these peptides.

2. Results and discussion

2.1. Mastoparan analogues, rational design and synthesis, activity and stability

Taking into account that our objective was to improve the stability of mastoparan and design analogues that enhance this stability, a human and mice serum stability assay with the original mastoparan was performed. Both results were exactly the same, observing the similarity when using both serums. Surprisingly at 6 h the intact mass of mastoparan (12.5%) was observed by MALDI-TOF. Lineal peptides are normally very attractive targets for peptidases and proteases. The most abundant peak observed by MALDI-TOF, apart from the original mass of our peptide, was the deletion of the isoleucine present at the *N*-terminal, which generated a peptide with a mass of 1366 Da (Fig. 1). It was not possible to calculate the half-life of the peptide by HPLC due to its almost coelution between the original peptide, mastoparan, and one of the resulting products after the incubation with serum. Neither could the half-life be calculated after using an isocratic gradient, especially in the presence of a low quantity of mastoparan.

The resulting peptide after the incubation with human serum (peptide 2) (Table 1) was synthesized in order to test its activity against several colistin-resistant *A. baumannii* strains. However, the MIC values for this peptide increased to very high levels (MIC values between 19.2 and 76.8 μM).

Taking into account, the information obtained in the stability assay and the fact that the resulting peptide is not active, ten peptides in addition to mastoparan (peptide 1) and the resulting peptide after the action of proteases and peptidases (peptide 2), were synthesized using solid-phase peptide synthesis (SPPS) (Table 1). They were obtained with a purity higher than 95%. Characterization of the peptides by HPLC is provided in the supporting information (SI). In order to enhance the stability of the peptide in human serum, several options were adopted. The first option was to introduce D-amino acids, resistant to proteases and peptidases, in the susceptible positions. Therefore the peptide with a D-isoleucine (peptide 3), another with a D-asparagine (peptide 4), and the peptide with both D-isoleucine and D-asparagine (peptide

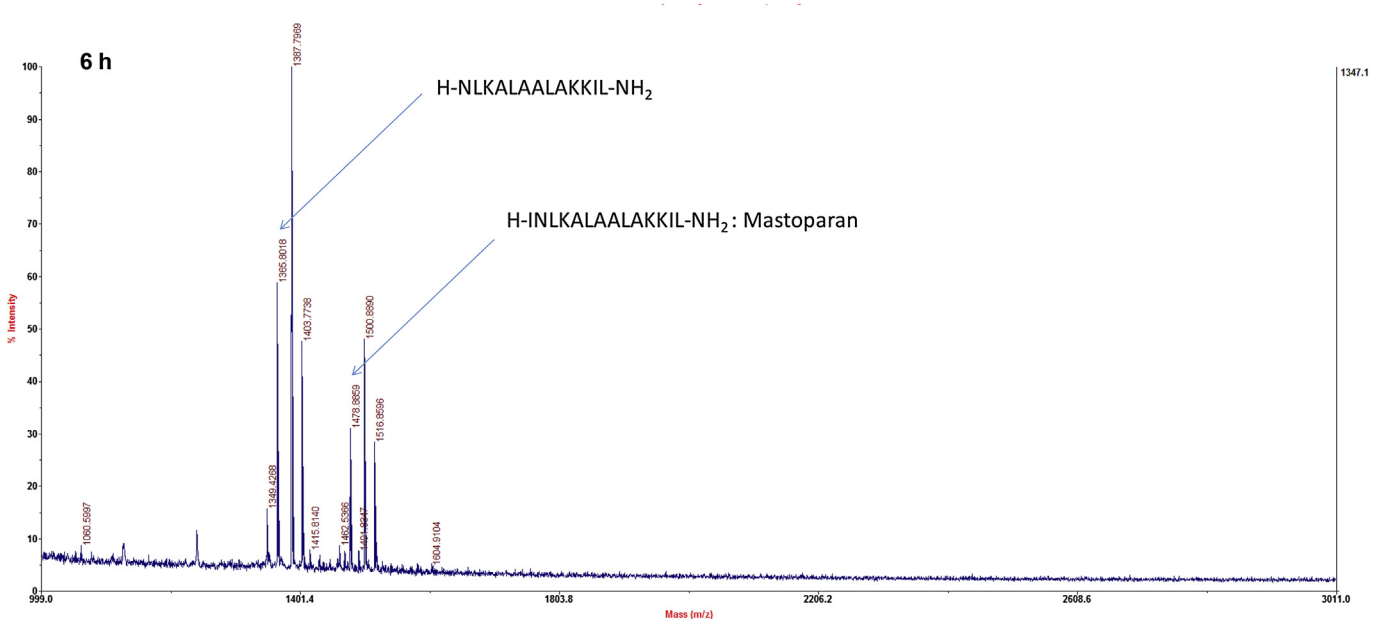

Fig. 1. MALDI-TOF MS spectra of mastoparan 6 h after incubation with human serum. The major compound is Peptide 2 (H-NLKALAALAKKIL-NH_2). (m/z) $[M+H]^+$ 1366, $[M+Na]^+$ 1388, $[M+K]^+$ 1404. The minor compound is mastoparan (H-INLKALAALAKKIL-NH_2). (m/z) $[M+H]^+$ 1480, $[M+Na]^+$ 1502, $[M+K]^+$ 1518.

Table 1
MIC and stability in human serum of different mastoparan analogs against colistin-resistant *A. baumannii* clinical isolates. D-amino acids are represented in lower case.

	Antimicrobial peptide	Minimal inhibitory concentration (μM) *A. baumannii* strains				Stability in human serum
		CR17	CR86	Ab11	Ab113	
1	**Mastoparan**	**2.7**	**2.7**	**2.7**	**2.7**	**12.5 % (6 h)**[a]
2	H-NLKALAALAKKIL-NH_2	19.2	38.4	76.8	76.8	93.47% (24 h)[b]
3	H-iNLKALAALAKKIL-NH_2	21.6	21.6	43.3	43.3	70.04% (24 h)[b]
4	H-lnLKALAALAKKIL-NH_2	21.6	43.3	86.5	173	71.98% (24 h)[b]
5	H-inLKALAALAKKIIL-NH_2	86.5	86.5	86.5	173	77.67% (24 h)[b]
6	H-LIKKALAALAKLNI-NH_2	173	173	173	173	12% (2 h)[a]
7	H-likkalaalaklni-NH_2	173	173	173	173	92.86% (24 h)[b]
8	H-inlkalaalakkil-NH_2	2.7	2.7	2.7	2.7	95.33% (24 h)[b]
9	H-INLKALAALAKKIL-$CH_2CH_2NH_2$	2.6	2.6	1.3	2.6	10.6% (6 h)[a]
10	H-LIKKALAALAKLNI-$CH_2CH_2NH_2$	42	84.1	84.1	42	8.5% (2 h)[a]
11	Ac-INLKALAALAKKIL-NH_2	10.5	21	42	42	74.34% (24 h)[b]
12	Gu-INLKALAALAKKIL-NH_2	2.6	2.6	2.6	2.6	79.20% (24 h)[b]

[a] Calculated using MALDI-TOF, taking into account same behaviour of both peptides.
[b] Calculated using HPLC by integrating peaks at time 0 h and 24 h. In bold Mastoparan activity and stability values

5) were synthesized.

Another common strategy followed when designing new peptidic drugs is to synthesize the retro (peptide 6), enantio (peptide 8) and retroenantio (peptide 7) versions of mastoparan. These three peptides have been found to be less cytotoxic than mastoparan [21]. The last strategy followed was to modify both the *C*- and *N*-terminus of the original mastoparan, without perturbing the original sequence of the peptide. Modifications at the *C*-terminal were performed using a special resin (1,2-diamino-ethane trityl), thereby obtaining an extra positive charge by the addition of an ethylamine moiety to the amide at the *C*-terminal of mastoparan (peptide 9) and retro-mastoparan sequences (peptide 10). The modifications at the *N*-terminal were performed by acetylating the free amine group (peptide 11), in which the action of the enzymes present in the serum diminished by the generation of steric hindrance of the acetyl group; however the peptide lost the positive charge present in the *N*-terminal. Another modification of the *N*-terminal was made by adding a guanidinium group, which also generates steric hindrance, in addition to maintaining the positive charge at the *N*-terminal.

These peptides were tested against four extended-resistant A. *baumannii* strains. The resistant profiles of these strains can be seen in (SI), highlighting that all of these strains were highly resistant to colistin. To test its increase in stability we also performed stability assays.

In terms of activity, the only peptides that maintained activity were peptides 8, 9 and 12 with the same MIC values for *A. baumannii* as those for mastoparan (2.6–2.7 μM). On comparing peptides 11 and 12 valuable information was obtained regarding the importance of conserving the positive charge in the *N*-terminal of these two peptides, with the MIC of the latter peptide increasing 8 and 16-fold, only with the removal of the positive charge. It is also important to highlight that with the change of only one single amino acid from the original sequence of mastoparan a high decrease in the antimicrobial activity of the peptide can be observed.

It was also of note that most of the peptides synthesized showed high stability in human serum compared to mastoparan. The peptides built with all D-amino acids (peptides 7 and 8), and peptide 2 showed very high stability with values of more than 90% after 24 h of incubation with human serum. Other peptides such as peptides 3, 4, 5, 11 and 12, also showed an increase in stability, reaching values of between 70 and 80% after 24 h. These values were calculated by integrating the peaks obtained in the HPLC spectra at 0 and 24 h. Other peptides synthesized that were built using L-amino acids in the most susceptible position were unprotected. Similar values compared to mastoparan were observed with peptide 9 (10.6% at 6 h), with even lower values found for peptides 6 and 10 with 12 and 8.5% at 2 h, respectively.

With the synthesis of all these peptides we found that most had high stability after 24 h in the presence of human serum, and some had the same activity as mastoparan, thereby suggesting that they may be useful in the treatment of infections caused by extended-drug resistant *A. baumannii* strains. We have also find out that the present of both an isoleucine and a positive charge in the *N*-terminal is really important for the activity of the compound according to the results obtained by the analogues synthesized. It is also possible to observe, that by introducing just one D-amino acid in the sequence a significant decrease in the activity of the peptides is observed, however when all the peptides are in the same L- or D-form the activity of these peptides is the same such us mastoparan and the retro version compared to its enantiomers. This fact could be affected by the loose of helicity when introducing a different amino acid form, and therefore decrease its activity. Another important feature to take into account before starting the *in vivo* assays is cytotoxicity; therefore some MTT assays of the most active compounds were performed using HeLa cells. Most of the active peptides showed similar cytoxicity values (SI), furthermore some hemolysis experiments were performed using mastoparan and the enantiomer version, in which low hemolysis was observed at MIC concentrations (SI). On review of the scientific literature, few effective peptide compounds have been described against colistin-resistant bacteria. Rodríguez-Hernández and colleagues [22] reported that the *in vitro* activity of cecropinA-melittin was similar against *A. baumannii* as the best peptides described in our study. However, optimization of the cecropinA-melittin was not performed. They also tested the *in vivo* activity with this peptide and only observed a local effect due to low *in vivo* stability [23]. Another peptide that has been described is api88 (Gu-ONNRP-VYIPRPRPPHPRL-NH_2) [24], which was found to be active against the most common Gram-negative pathogens such as *E. coli*, *P. aeruginosa*, *K. pneumoniae* or *A. baumannii* with MIC values below 1.8 μM. It was optimized in terms of activity and afterwards tested *in vivo*, showing good *in vivo* response against *E. coli*. Two peptides isolated from frog-skin secretions ([E4K]alyteserin-1c [GLKEIFKAGLGSLVKGIAAHVAS-NH_2], [D4K]B2RP [GIWK-TIKSMGKVFAGKILQNL-NH_2]) have been tested against both colistin-susceptible and -resistant *Acinetobacter* species and showed similar values for all the strains tested (1.7–7.1 μM). Nonetheless, the cytoxicity and stability of these peptides were not optimized, and therefore, their activity *in vivo* may actually be very low [25].

2.2. Mechanism of action of mastoparan analogues

2.2.1. Analysis of the leakage

Leakage assays were performed using two different membrane mimetics, a negatively charged membrane mimicking bacterial membranes, and a neutral membrane. Negatively charged membranes are composed by phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidylglycerol (PG) 63:14:23. Although this is the composition of *P. aeruginosa*, its high similarity with *A. baumannii* allows it to also be used [26]. The concentrations of peptides used to test their ability to release carboxyfluorescein from the liposome were 0.1, 0.25, 0.5, 1, 10 and 50 μM. Fig. 2(A) and Table S4-A (SI) shows the percentage of release of each peptide at the concentrations mentioned above. No release was observed at concentrations below 1 μM. At 10 μM some differences were observed between the peptides, and most of the active peptides against highly-resistant strains of *A. baumannii* showed a higher ability to release the fluorophore from the negative liposomes. Peptide 8 reached 87% of release followed by mastoparan and peptide 12, with 72 and 60%, respectively. These three peptides were also the most active at low MIC values (2.6–2.7 μM). Despite the different MIC values of peptides 9 and 11 (peptide 9, 1.3–2.6 μM and peptide 11, 10.5–42 μM) these peptides share the same percentage of release (53%). The next three peptides, peptides 2, 3 and 4, with high MIC values of greater than 19.2 μM, also had similar release values with 35, 33 and 37%, respectively. All these peptides released the carboxifluorescein present inside liposome at 50 μM.

The last four peptides (5,6,7 and 10) were the least active in terms of both *in vitro* activity against *A. baumannii* and carboxifluorescein release. For peptide 6 the percentage of release at 10 μM was 14%, rising to up to 51% at 50 μM. The last three peptides, peptides 5, 7 and 10, had MICs greater than 42 μM and all showed very low release values of 10% at 10 μM, At 50 μM the only peptide to show a slight increase in release was peptide 5 which increased up to 20% while these values remained at around 10% for the other two peptides.

The values observed for the neutral liposomes (EPC/cholesterol, 5:1) used as bacteria models can be divided into four main groups as it can observed in both Fig. 2(B) and Table S4-B (SI). The first group was composed by the best compound, peptide 8, with a 65% of carboxyfluorescein released at 50 μM. The second group was composed by mastoparan and peptides 2, 3, 4, 9, 11 and 12, with the percentage of release ranging from 44% to 36% at 50 μM (Fig. 2B). Peptides 6 and 7, belonged to the third group, having percentages of release of 19 and 17%, respectively at 50 μM. The last group included the peptides 10 and 5, which showed almost no activity at either 10 μM or 50 μM, with values of less than 10%. On analysing the values obtained, it was found that the most active compounds (lower MIC values) also had the highest percentage of carboxyfluorescein release. It was also of note that, the release vales

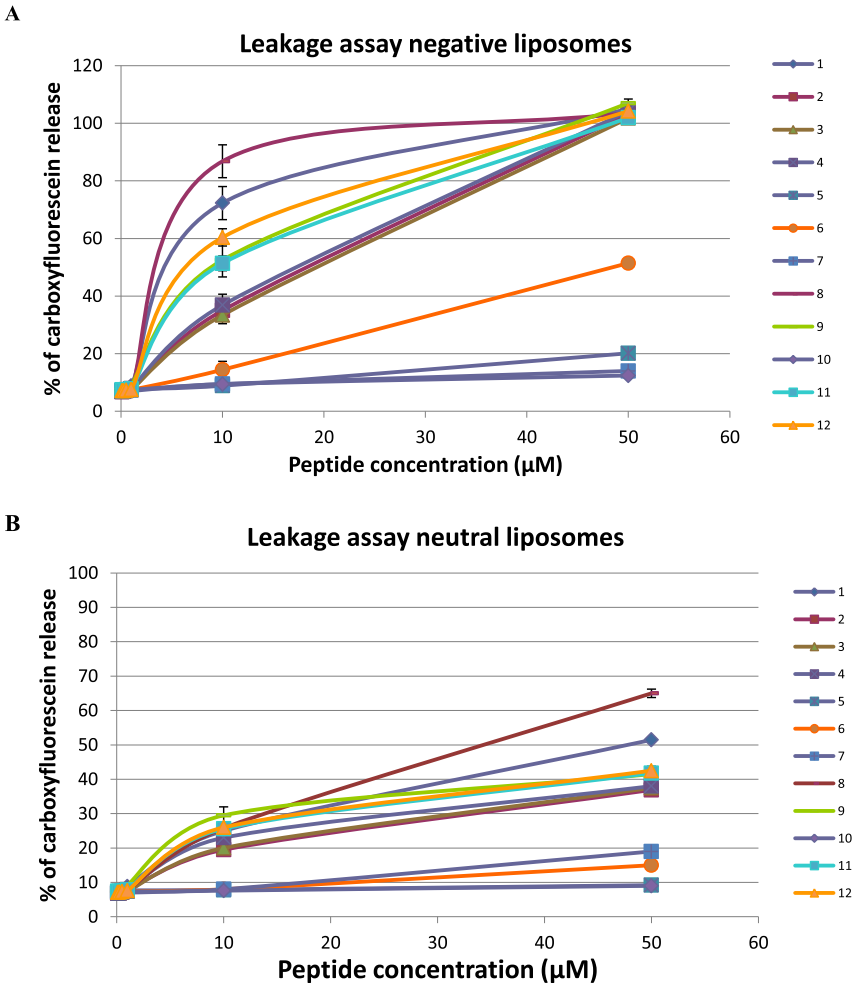

Fig. 2. **A**) Percentage of carboxyfluorescein release of all the mastoparan analogues at different concentrations using negative liposomes. **B**) Percentage of carboxyfluorescein release of all the mastoparan analogues at different concentrations using neutral liposomes.

obtained when incubating these peptides with negative liposomes were higher compared to neutral liposomes due the cationic nature of the peptides synthesized. The different interaction between positively charged peptides and negative or neutral liposomes may explain the different values observed. The leakage values of the lactoferrin analogues using liposomes mimicking Gram-negative membranes were similar to those presented in our study [27]. A similar effect was observed with some peptides of the mastoparan family. However, the authors did not compare the activity of the peptides with the leakage, but rather they only correlated the number of cationic charges with the leakage [28]. In contrast, the results of our study showed different leakage despite the presence of the same number of positive charges.

2.2.2. Transmission electron microscopy

The effect of mastoparan and peptide 8 on the cell morphology of extended-resistant *A. baumannii* was also investigated by transmission electron microscopy (TEM). Untreated cells of *A. baumannii* grown in LB (Luria Broth) medium showed a normal morphology with no structural damage to any of the membranes when grown in the absence of the peptide (Fig. 3A). After 1 h of incubation with mastoparan and peptide 8 (at the MIC for each peptide), the bacteria showed considerable damage with fractures in the membrane in the bacteria incubated with either mastoparan or peptide 8 (Fig. 3B and C). Although with the information obtained with the TEM studies we cannot confirm that the mechanism of action of these peptides is due to a membrane disruption, our results, together with the data obtained with the leakage experiments, as well as previous reports on the mechanism of action of the mastoparan peptide family suggest that membrane disruption is the mechanism by which these peptides kill bacteria. Using the peptides Gramicidin S/PGLa and NK-2, respectively, Hartman et al. [29] and Hammer *at al* [30]. observed the formation of membrane blebs, electron-dense surfaces or membrane ruffling in *E. coli*, whereas the formation of holes was observed in our study on incubating *A. baumannii* with mastoparan. These different effects of the AMPs demonstrate that the mechanism observed depends on the type of pathogen, the peptide or the concentration used.

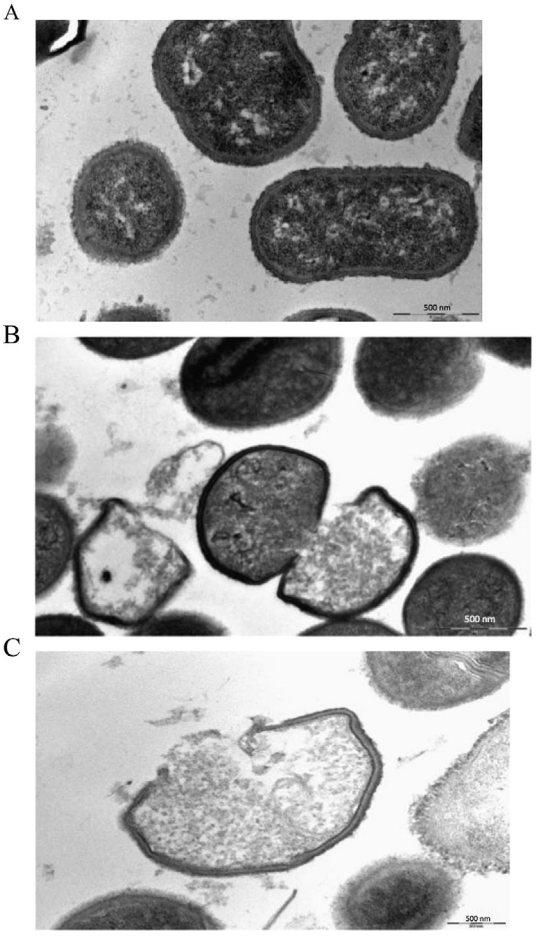

Fig. 3. A) TEM of untreated colistin-resistant *A. baumannii* cells. **B**) TEM of colistin-resistant *A. baumannii* cells incubated with mastoparan. **C**) TEM of colistin-resistant *A. baumannii* cells incubated with mastoparan enantiomer.

3. Conclusion

In the present study we optimized an initial hit (mastoparan) in an attempt to overcome one of the most important drawbacks of drug peptides which is their low stability in human serum. We therefore chose the option to protect the susceptible points of proteolysis of the molecule. Other options may increase the stability such as polyethyleneglycol (PEG) [31], the use of lipids for drug delivery of the peptides [32], the use of nanoparticules in order to achieve a longer life time [33] or the use of dendrimers [34]. The mastoparan family is a very promising group of potential new drugs. In addition to antiviral activity [35] our results show that mastoparan and some of its analogues may be potential antibacterial agents to treat infections caused by multi-, extended- and pan-drug resistant A. *baumannii* which have the ability to overcome all the antibiotics administered. Their good *in vitro* activity against this microorganism allows very high stability in human serum and moderate toxicity in HeLa cells. However, further *in vivo* studies involving the best candidates should be performed.

4. Materials and methods

4.1. Bacterial strains

The four *A. baumannii* strains used in this study were clinical isolates from the Hospital Virgen del Rocio in Sevilla (Spain).

4.2. Susceptibility testing

The MICs of all the peptides for the *A. baumannii* strains were determined with the microdilution method following the CLSI guidelines [36]. The concentrations ranged from 187.5 μM to 0.34 μM. The E-test (Biomerieux, Marcyl'Etoile, France) was used to determine the susceptibility of the strains used in this study to a group of antibacterial agents.

4.3. Materials

The synthesis of all the peptides except the two with a positive charge in the *C*-terminal was made using Rink amide-Chemmatrix resin purchased from PCAS BioMatrix (Quebec, Canada). The other two peptides were synthesized using 1,2-diamino-ethane trityl resin from Novabiochem (Merck) (Darmstadt, Germany). The coupling reagents used were: 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) from Albatros Chem, Inc. (Montreal, Canada); Trifluoroacetic acid (TFA) was from Scharlab S.L. (Barcelona, Spain). Piperidine, dimethylformamide (DMF), dichloromethane (DCM) and acetonitrile (MeCN) were from SDS (Peypin, France); N,N-diisopropylethylamine (DIEA) was obtained from Merck (Darmstadt, Germany) and Tri-isopropylsilane (TIS) was from Fluka (Buchs, Switzerland).

4.4. Peptide synthesis

Peptides were synthesized by solid-phase peptide synthesis (scale: 100 μmol) using the 9-fluorenylmethoxycarbonyl/tert-butyl (Fmoc/^{t}Bu) strategy. N$^{\alpha}$-Fmoc-protected amino acids (300 μmol)/TBTU (300 μmol), and DIEA (600 μmol) (3eq:3eq:6eq) were used. The Fmoc protecting group was cleaved by treatment with a solution of 20% piperidine in DMF. For the acetylated peptides 50eq of Ac_2O and 50eq of DIEA in DCM were used. For guanidination, 5 eq of 1,3-Di-Boc-(trifluoromethylsulfonyl) guanidine and 5 eq of triethyl amine in DCM were used. Peptides were cleaved from both resins using 95% TFA, 2.5% TIS, 2.5% water for 3 h. The peptides have been cleaved using TFA and the HPLC solvents contain also TFA, this have been removed after liophilization. The peptides are all in their trifluoroacetate form as counter ion. The peptides were analysed at $\lambda = 220$ nm by analytical HPLC [Waters Alliance 2695 separation module equipped with a 2998 photodiode array detector, Sunfire C_{18} column (100 mm × 4.6 mm x 3.5 mm, 100 Å, Waters), and Empower software; flow rate = 1 mL/min. The peptides were then purified by semi-preparative HPLC [Waters 2700 Sample Manager equipped with a Waters 2487 dual λ absorbance detector, a Waters 600 controller, a Waters fraction collection II, a Symmetry C_{18} column (100 mm × 30 mm, 5 mm, 100 Å, Waters) and Millenium chromatography manager software]. Flow rate = 15 mL/min; solvents: A = 0.1% trifluoroacetic acid in water, and B = 0.05% trifluoroacetic acid in acetonitrile. Peptides were characterized by MALDI-TOF mass spectrometry (Voyager-DE RP MALDI-TOF, PE Biosystems with a N2-laser of 337 nm) and a high resolution ESI-MS model (LTQ-FT Ultra, Thermo Scientific).

4.5. Peptide human serum stability

The peptides were incubated at 37 °C in the presence of 100% human serum (from human male AB plasma). At different times, 200 μL aliquots were extracted and serum proteins were precipitated by the addition of 400 μL of acetonitrile at 4 °C to stop degradation (2:4, v/v). After 30 min at 4 °C, the samples were centrifuged at 10,000 rpm (9300 × g) for 10 min at 4 °C. The supernatant was analysed by HPLC (flow = 1 mL/min; gradient = 0–100% B in 8 min; A = 0.045% trifluoroacetic acid in H_2O, B = 0.036% trifluoroacetic acid in acetonitrile). The fractions were also analysed by MALDI-TOF mass spectrometry (Voyager-DE RP MALDI-TOF, PE Biosystems with a N2-laser of 337 nm).

4.6. Leakage assay

Aliquots containing the appropriate amount of lipid in chloroform/methanol (1:1, v/v) were placed in a test tube, the solvents were removed by evaporation under a stream of O_2-free nitrogen, and finally traces of solvents were eliminated under vacuum in the dark for more than 3 h. Afterwards, 1 mL of buffer containing 10 mM HEPES, 100 mM NaCl, 0.1 mM EDTA, pH 7.4 buffer and carboxyfluorescein at a concentration of 40 mM was added, and multilamellar vesicles were obtained. Large unilamellar vesicles (LUVs) with a mean diameter of 200 nm were prepared from the multilamellar vesicles by the LiposoFast device from Avestin, Inc., using polycarbonate filters with a pore size of 0.2 μm (Nuclepore Corp). Breakdown of the vesicle membrane led to content leakage, i.e., carboxyfluorescein fluorescence. Non-encapsulated carboxyfluorescein was separated from the vesicle suspension through a Sephadex G-25 filtration column eluted with buffer containing 10 mM HEPES, 150 mM NaCl, and 0.1 mM EDTA, pH 7.4. Leakage of intraliposomal carboxyfluorescein was assayed by treating the probe-loaded liposomes (final lipid concentration, 0.125 mM) with the corresponding amount of peptide in Costar 3797 round-bottom 96-well plates, with each well containing a final volume of 100 μl. The micro titre plate was incubated at RT for 1 h to induce dye leakage. Leakage was measured at various peptide concentrations. Changes in fluorescence intensity were recorded using the FL600 fluorescence microplate reader with excitation and emission wavelengths set at 492 and 517 nm, respectively. Total release was achieved by adding Triton X-100 to a final concentration of 1% v/v to the microtitre plates. Fluorescence measurements were initially made with probe loaded liposomes, followed by the addition of the peptide and, finally the addition of Triton X-100 to obtain 100% leakage. The results were expressed as percentage of carboxyfluorescein released relative to the positive control (Triton X-100).

4.7. Cytotoxicity (MTT) assay

HeLa cells were used for these experiments. Their doubling time and the lineal absorbance at 570 nm were taken into an account for seeding purposes. Cell viability in the presence of peptides was tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For each assay, 5×10^3 HeLa cells were seeded on a 96-well plate (NalgeNunc) and cultured for 24 h. Samples were added at concentrations ranging from 0.1 μM to 500 μM depending on the peptide. Cells were incubated for 24 h at 37 °C under a 5% CO_2 atmosphere. After 20 h, medium with compounds was removed, and MTT was added to a final concentration of 0.5 mg/mL. MTT was incubated for a further 4 h, and the medium was then discarded. DMSO was added to dissolve the formazan product, and absorbance was measured at 570 nm after 15 min. Cell viability percentages were calculated by dividing the absorbance value of cells treated with a given compound by the absorbance of untreated cells.

4.8. Transmission electron microscopy (TEM)

Bacteria were grown in LB media and in mid-log exponential were incubated with MIC (2.7 μM) concentrations with mastoparan and mastoparan enantiomer for 1 h at 37 °C. A control without peptide was also performed. After the incubation, centrifugation at 3500 rpm 4 °C was done. The pellets were then fixed for 1 h with 2% of gluteraldehyde, washed three times with water and then fixed again with 1% of OsO_4. The post-fixation positive stain was carried out with 3% of uranyl acetate acqueous solution during 1.5 h, after which graded ethanol series (30, 50, 70, 90 and 100%) were carried out every 15 min for dehydration purposes. The samples were embedded in an epoxy resin. A Tecnai Spiritmicroscope (EM) (FEI, Eindhoven, The Netherlands) equipped with a LaB6cathode was used. Images were acquired at 120 kV and room temperature with a 1376 x 1024 pixel CCD camera (FEI,Eindhoven, The Netherlands).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://

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